Supplemental Experimental Procedures

Animals

Animals were maintained on 12 h:12 h light/dark cycle with food and water available *ad libitum*. All surgical and behavioral procedures in mice were performed in compliance with NIH guidelines and protocols approved by the IACUC of the Rockefeller University and of the Harvard University. The Gal::Cre BAC transgenic line (STOCK Tg(Gal-cre)KI87Gsat/Mmucd, 031060-UCD) was imported from the Mutant Mouse Regional Resource Center and is described (Wu et al., 2014).

iDISCO+ sample processing

Modifications and continuous updates to the protocol can be found at http://www.idisco.info.

iDISCO+ protocol

Sample collection

Adult mice were euthanized with a rising gradient of CO2 and fixed with an intracardiac perfusion of 4% PFA in PBS. All harvested samples were post-fixed overnight at 4°C in 4% PFA in PBS.

Fixed samples were washed in PBS for 1h twice, then in 20% methanol (in ddH2O) for 1h, 40% methanol for 1h, 60% methanol for 1h, 80% methanol for 1h, and 100% Methanol for 1h twice. Samples were then bleached with 5% H2O2 (1 volume of 30% H2O2 for 5 volumes of methanol, ice cold) at 4°C overnight. After bleaching, samples were re-equilibrated at room temperature slowly and re-hydrated in 80% methanol in

H2O for 1h, 60% methanol / H2O for 1h, 40% methanol / H2O for 1h, 20% methanol / H2O for 1h, and finally in PBS / 0.2% TritonX-100 for 1h twice.

Pre-treated samples were then incubated in PBS / 0.2% TritonX-100 / 20% DMSO / 0.3M glycine at 37°C for 36h, then blocked in PBS / 0.2% TritonX-100 / 10% DMSO / 6% Donkey Serum at 37°C for 2 days. Samples were then incubated in primary antibody dilutions (see table S4) in PBS-Tween 0.2% with Heparin 10μg/mL (PTwH) / 5% DMSO / 3% Donkey Serum at 37°C for 4 days (half brains) or 7 days (whole brains). Samples were then washed in PTwH for 24h (5 changes of the PTwH solution over that time), then incubated in secondary antibody dilutions (e.g. donkey anti-rabbit-Alexa647 at 1/500th in PTwH / 3% Donkey Serum) at 37°C for 4 days (half brains) or 7 days (whole brains). Samples were finally washed in PTwH for 1d before clearing and imaging.

Sample clearing:

Immunolabeled brains were cleared with the following modified version of the original 3DISCO protocol (Ertürk et al., 2012). Samples were dehydrated in 20% methanol (in ddH2O) for 1h, 40% methanol / H2O for 1h, 60% methanol / H2O for 1h, 80% methanol / H2O for 1h, and 100% Methanol for 1h twice. Samples were incubated overnight in 1 volume of Methanol / 2 Volumes of Dichloromethane (DCM, Sigma 270997-12X100ML) until they sank at the bottom of the vial (Plastic Eppendorf tubes are used throughout the process). The methanol was then washed for 20min twice in 100% DCM. Finally, samples were incubated (without shaking) in DiBenzyl Ether (DBE, Sigma 108014-1KG) until clear (about 30min) and then stored in DBE at room temperature. Organic solvents should be handled under a chemical hood, and disposed according to local health and safety regulations.

Light sheet imaging

Cleared samples were imaged in sagittal orientation (right lateral side up) on a light-sheet microscope (Ultramicroscope II, LaVision Biotec) equipped with a sCMOS camera (Andor Neo) and a 2X/0.5 objective lens (MVPLAPO 2x) equipped with a 6mm working distance dipping cap. Version v144 of the Imspector Microscope controller software was used. The microscope is equipped with LED lasers (488nm, 561nm and 640nm) with 3 fixed light sheet generating lenses. Scans were made at the 0.8X zoom magnification (1.6X effective magnification), with a light sheet numerical aperture of 0.1. Emission filters used are 525/50, 595/40 and 680/30. The samples were scanned with a step-size of 3μm using the continuous light sheet scanning method with the included contrast blending algorithm for the 640nm and 595nm channels (20 acquisitions per plane with a 50ms exposure), and without horizontal scanning for the 480nm channel (50ms exposure). To speed up the acquisitions, both channels where acquired in two separate scans. To account for micro-movements of the samples that may occur between the scans, a 3D image affine registration was performed to align both channels using ClearMap.

Image processing and analysis

For display purposes in the figures, a gamma correction of 1.47 was applied on the raw data obtained from the light-sheet microscope (but not on the density maps, which are linearly scaled). Maximum projections were performed using ImageJ (NIH,

http://imagej.nih.gov/ij/). Imaris (Bitplane, http://www.bitplane.com/imaris/imaris) was used for the manual 3D annotations in figure 2A, 3B,C and movies. Imaris was also used to generate the orthogonal projections of the unprocessed data in all figures.

ClearMap analysis

All analysis and quantifications where performed with the newly developed open source ClearMap software as shown in the example scripts (Supplementary data 2), and the associated documentation. The latest version of ClearMap can be obtained from http://www.idisco.info.

The cell detection is tailored for cell nuclei and uses a background subtraction via morphological opening, followed by a sequence of filters, morphological operations and a 3D peak detection. The peaks are used as seeds for a watershed on the foreground and the resulting shapes filtered by volume to retain only genuine cells (Figure S3A and B). To improve the consistency of the cell detection algorithm, we pre-processed the light sheet images by correcting illumination along the y axis (orthogonal to the direction of illumination) to account for light loss due to the objective lens vignetting and Gaussian shape of the light sheet beam (Figure S4A). To tune the algorithm and filters, two users manually annotated all the cells in a substack from our datasets (812x812x195μm), and iterated the filter's parameters to obtain the best overlap between the manual and automatic annotations. With a threshold of 6 voxels for the cell size, the algorithm detected 725 cells, 83% of which were independently annotated by user 1 and 75% by user 2, which is similar to the overlap between user 1 and user 2 (79%) (Supplementary figure 3C). We chose for subsequent studies a higher threshold of 20 voxels for the cell

size, which insures an agreement rate of over 99% between users and the algorithm. We verified that the chosen resolution of 4.06x4.06x4.5μm/pixel used did not affect the number of cells imaged nor the accuracy of the cell detection by imaging the same sample at the 1.6X magnification used throughout the study and a 4X higher magnification. The cell detection algorithm detected cells in similar numbers and equally well in both configurations (2634 cells detected at 1.6X in the region matching the 4X scan, versus 2723 cells detected in the 4X scan) (Figure S5).

The settings used for analyzing the data were as follow: the background was removed by subtraction of the morphological opened image with a disk shape structure element with main axis of 7 pixels of diameter. Cells were detected from peaks and subsequent watershedding, removing background pixels below an intensity cutoff of 700 (700 is the rounded average value of the background intensity based on unfiltered raw intensities, see Figure S3B) and selecting cells with sizes between 20 to 500 voxels. Density maps were generated by summing spheres of 375μm diameter (= 15 pixels) and uniform intensity centered on each cell. This value was chosen to help representing regions with very low cell counts, such as the thalamus. However, smaller spheres can also be used and yield very similar results. Intensity-weighted density maps and statistics are presented in the figures. Samples were registered using the average autofluorescence STPR brain (Kim et al., 2015) registered to the Allen Brain Institute 25μm map, and companion annotation map (http://alleninstitute.org/). Integrated intensities (weighted counts) were calculated by summing the peak intensity values for each detected cell center within a given region (spheres for voxelization or labeled region for annotated counts).

Statistics

Cell counts or intensities of each sample in considered regions or annotated brain areas between different groups were compared using the independent two sample student t-test assuming unequal variances. Statistical tests were performed numerically using the SciPy statistics library (http://www.scipy.org/). Using ClearMap as a discovery tool p-values were corrected for multiple comparison to q-values to control for false-discovery rate (Benjamini and Hochberg, 1995).

MRI scanning

Wild type adult littermates were transcardially perfused with 0.9% saline and 4% PFA. The brains were dissected, post-fixed, cut sagitally in half (to fit in the MRM scanner tube without deformation) and stored in PB until imaging.

All MRM scans were performed on a 9.4T Bruker vertical bore magnet. An actively shielded gradient coil set was used with gradient strenghts of up to 100G/cm. A RF birdcage coil with 10mm inner diameter was used to scan the specimens. Before MRM scanning the mouse brains were washed in PBS and carefully dried to remove remainder PBS or fixative. It is then placed in a 10mm polyethylene tube filled with Fomblin (perfluoropolyether, Ausimont, Morristown, NJ). Fomblin was used to seal the specimen to prevent dehydration and as the same time to prevent susceptibility effects. Fomblin does not produce any MR signal and therefor was a better choice as compared to formalin for maximizing signal dynamic range. The mouse brains that we extracted were around 10mm in width and fit snug in the tube. Additional Fomblin-soaked gauze padding was

added to insure stability. For the structural MR acquisition protocol we used a 3D RARE (Rapid Acquisition Relaxation Enhanced) sequence. The scanning parameters were as follows: TR=1900ms, TE=17.5ms with a RARE factor of 4. Matrix size was 512x512x512 with a field of view of 12.8mm resulting in an isotropic resolution of 25µm. We obtained 2 averages.

Haloperidol injections

Adult (10 weeks old) male littermate mice were individually housed for 5 days and on the 6-th day were injected i.p. either 1 mg/kg Haloperidol or vehicle (0.3%-TWEEN-80 in 0.9% saline). The mice were euthanized 3 hours after injection, transcardially perfused with 0.9% saline and 4% PFA. The brains were dissected out, post-fixed overnight in 4% PFA at 4°C and washed in PB until entering the iDISCO+ pipeline.

New environment exploration

Mice were anesthetized with a mixture of Ketamine / Xylazine (Ketamine at 0.1mg/g, Xylazine at 0.01mg/g) and their whiskers were trimmed or left intact (sham operation for the intact-whiskers group). Mice were then left to recover single-housed in an empty cage with access to food and water for 24h in the dark. Mice where then transferred to a new cage containing an enriched environment crowded with cardboard tubes and wire tube racks for 1h to explore. Mice were then promptly anesthetized, perfused and processed according to the iDISCO+ protocol.

Viral injections

Viral tracing experiments were performed in Gal::Cre mice at ~8-12 weeks of age. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine via intra-peritoneal injection. 500 nl of AAV1-EF1a-DIO-hChR2(H134R)eYFP (UPenn Vector Core) were injected into the right medial preoptic area using a Nanoject II injector (Drummond Scientific). The coordinates were: AP Bregma: 0.0mm, ML +0.5mm; DV: -5.05mm. Two weeks later, mice underwent behavioral testing.

Parental behavior assay

Parental behaviour assays were performed in sexually naive Gal-cre females at ~8-12 weeks of age, 2 weeks after viral injection. Animals were individually housed for one week before testing. On testing day, animals were habituated to the testing arena for 30min. Two C57BL/6J pups (age P1-3) were then introduced into the cage and placed at the farthest corners from the resident's nest. A timer was started at the first retrieval of a pup to the nest. All mice included in this study showed the following stereotypic sequence of maternal behaviors: pup grooming and licking, nest building, retrieval and crouching (assuming of a nursing-like posture over the pups). 90 min after retrieval, mice were deeply anesthetized with isoflurane and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA, in PBS). Brains were quickly removed and post-fixed in 4% PFA overnight. Only mice that retrieved at least one pup to the nest were included in this study (~80% of tested animals).

Infanticide behavior assay

Infanticide behavior assays were performed in sexually naive C57BL/6J males at ~8-12 weeks of age that had not been exposed to pups. Animals were individually housed for one week before testing. On testing day, animals were habituated to the testing arena for 2h with hydrogel and food in the cage. Two C57BL/6J pups (age P1-3) were then introduced into the cage and placed at the farthest corners from the resident's nest. The first olfactory investigation marked the beginning of the assay, which was continued if the resident attacked at least one pup. When a pup was attacked, the assay was ended immediately and the wounded pup was euthanized. 90 min after first olfactory investigation, infanticidal males were euthanized, perfused and their brains collected and post-fixed for iDISCO treatment (see 'Parental behavior assay').

Supplementary Table 4: Primary antibodies used in this study. Related to Figure 2.

Antigen	Host	Vendor	Reference	Dilution
c-Fos	Rabbit	Santa Cruz	sc-52	1/500
Arc	Rabbit	Synaptic Systems	156003	1/1000
p-S6	Rabbit	Life Technologies	44923G	1/300
Egr1	Rabbit	Santa Cruz	sc-110	1/200
Npas4	Rabbit	Gift from Yingxi Lin, MIT		1/5000
GFP	Chicken	Aves	GFP-1020	1/2000
TH	Rabbit	Millipore	AB-152	1/500